REMARKS / ARGUMENTS

1. The Amendments to the Specification

The specification has been amended to correct a typographical error in the numbering of page 40. No new matter has been added.

2. The Amendments to the Claims

Before this Amendment, claims 1-65 were pending. Upon entry of the present amendments, claims 1, 2, 5-11, 26-31 and 47-50 will be pending and under active consideration. Claims 3, 4, 12-25, 32-46 and 51-65 have been canceled without prejudice, as being drawn to an invention non-elected with traverse in the Applicant's Response to the Examiner's Office Action dated November 3, 2004. The Applicant expressly reserves all rights to prosecute claims drawn to any subject matter removed by claim cancellation or by claim amendment made herein in a subsequent continuation application.

3. Restriction Requirement

The Examiner acknowledges the Applicant's election with traverse of Group I and has now made the restriction requirement final; claims 3, 4, 12-25, 32-46 and 51-65 were withdrawn from further consideration, as being directed to non-elected subject matter. The Applicant maintains that the restriction requirement should be withdrawn, and earnestly requests reconsideration of the finality of the restriction requirement.

4. Objection to Specification

The Examiner has objected to the specification on grounds that the third page of the claims is incorrectly numbered.

The specification has been amended to correct the error. It is respectfully submitted that the objection is thus overcome. Reconsideration and withdrawal of the objection to the specification are therefore respectfully requested.

5. Double Patenting

Claims 10 and 11 are objected to under 37 CFR 1.75 as allegedly being substantial duplicates of claim 6. The Applicant respectfully disagrees. Claims 10 and 11 each expressly recite an additional feature of an embodiment of the invention that is not recited in claim 6.

Claim 6 is drawn to a vaccine comprising:

a) a vector capable of expressing a recombinant DNA inserted into said vector such that a recombinant protein is expressed when said vector is provided in an appropriate host; and

b) the recombinant DNA inserted into said vector wherein said DNA is selected from the group consisting of:

i) a recombinant DNA that encodes a protein having an amino acid sequence as shown in SEQ. ID. NO. 3;

ii) a recombinant DNA that encodes a protein having an amino acid sequence as shown in SEQ. ID. NO. 5;

iii) a recombinant DNA that encodes a protein having an amino acid sequence as shown in SEQ. ID. NO. 7;

iv) a recombinant DNA that encodes a protein having an amino acid sequence as shown in SEQ. ID. NO. 9;

v) a recombinant DNA that encodes a protein having an amino acid sequence as shown in SEQ. ID. NO. 11; and

vi) any portion of said DNA above that encodes a protein fragment that is greater than 25 amino acids.

Claim 10 recites an additional feature of one embodiment of the vaccine of claim 6, namely, that the vaccine is administered into said host by a method selected from the group consisting of:

a) intramuscular injection;

b) intravenous injection; and

c) gene gun injection.

This limitation, which is not recited in claim 6 (from which claim 10 depends) limits the scope of the vaccine to a vaccine that is administered by a method selected from the recited group of three administration methods.

Claim 11 recites an additional feature of one embodiment of the vaccine of claim 10, namely, that the host into which the vaccine is administered is a dog. This limitation, which is not recited in claims 6 or 10 (from which claim 11 depends) limits the scope of the vaccine to a vaccine that is administered to a host that is a dog.

Since each of claims 10 and 11 recite a limitation that is not recited in claim 6, and since that limitation further limits the scope of the claimed vaccine, it is respectfully requested that the objection to claims 10-11 on the grounds of double-patenting be reconsidered and withdrawn.

6. **The Rejections**

   a. **Examiner's Rejections Under 35 U.S.C. § 112 (First Paragraph) Should Be Withdrawn**

Claims 1, 2, 5-11, 26-31 and 47-50 have been rejected under 35 U.S.C. §112, first paragraph, as containing subject matter which was not described in the specification in such a way as to enable one skilled in the art to which it pertains, or with which it is most nearly connected, to make and/or use the invention. The Applicant respectfully disagrees with the rejection.

The test for enablement is whether the disclosure, when originally filed, contained sufficient information regarding the subject matter of the claims as to enable those of ordinary skill in the pertinent art to make and use the invention. The standard is whether the experimentation necessary to practice the invention is undue or unreasonable. In re Wands, 858 F.2d 731, 737, 8 USPQ2d 1400, 1404 (Fed. Cir. 1988). *See also* U.S. v. Telectronics, Inc., 857 F.2d 778, 785, 8 USPQ2d 1217, 1223 (Fed. Cir. 1988) ("The test of enablement is whether one reasonably skilled in the art could make or use the invention from the disclosures in the patent coupled with information known in the art without undue experimentation.") (emphasis added).

The fact that experimentation may be complex does not necessarily make it undue, if the art typically engages in such experimentation. In re Certain Limited-Charge Cell Culture Microcarriers, 221 USPQ 1165, 1174 (Int'l Trade Comm'n 1983), *aff'd. sub nom.*, Massachusetts Institute of Technology v. A.B. Fortia, 774 F.2d 1104, 227 USPQ 428 (Fed. Cir. 1985). *See also* In re Wands, 858 F.2d at 737, 8 USPQ2d at 1404. Thus, the test of enablement is not whether any experimentation is necessary, but whether, if experimentation is necessary, it is undue. In re Angstadt, 537 F.2d 498, 504, 190 USPQ 214, 219 (C.C.P.A. 1976).

There are many factors to be considered when determining whether there is sufficient evidence to support a determination that a disclosure does not satisfy the enablement requirement and whether any necessary experimentation is "undue." These factors include, but are not limited to: the breadth of the claims; the nature of the invention; the state of the prior art; the level of one of ordinary skill; the level of predictability in the art; the amount of direction provided by the inventor; the existence of working examples; and the quantity of experimentation needed to make or use the invention based on the content of the disclosure. In re Wands, 858 F.2d 731, 737, 8 USPQ2d 1400, 1404 (Fed. Cir. 1988) (reversing the PTO's determination that claims directed to methods for detection of hepatitis B surface antigens did not satisfy the enablement requirement). The Examiner's analysis must consider all the evidence related to each of the Wands factors, and any conclusion of non-enablement must be based on the evidence as a whole. In re Wands, 858 F.2d at 740, 8 USPQ2d at 1407.

The enablement requirement is often more indulgent than the written description requirement. The specification need not explicitly teach those in the art to make and use the invention; the requirement is satisfied if, given what they already know, the specification teaches those in the art enough that they can make and use the invention without "undue experimentation." Genentech, Inc. v. Novo Nordisk, A/S, 108 F.3d 1361, 1365, 42 USPQ2d 1001, 1004 (Fed. Cir. 1997); In re Vaeck, 947 F.2d 488, 495, 20 USPQ2d 1438, 1444 (Fed. Cir. 1991). Indeed, a patent need not teach, and preferably omits, what is well known in the art. In re Buchner, 929 F.2d 660, 661, 18 USPQ2d 1331, 1332 (Fed. Cir. 1991); Hybritech, Inc. v. Monoclonal Antibodies , Inc., 802 F.2d 1367, 1384, 231 USPQ 81, 94 (Fed. Cir. 1986), *cert. denied*, 480 U.S. 947 (1987); Lindemann Maschinenfabrik GMBH v. American Hoist & Derrick Co., 730 F.2d 1452, 1463, 221 USPQ 481, 489 (Fed. Cir. 1984).

Further, "the law makes clear that the specification need teach only one mode of making and using a claimed composition." Amgen, Inc. v. Hoescht Marion Roussel, Inc., 126 F. Supp. 2d 161, 57 USPQ2d 1516 (D. Mass. 2001) (citing Johns Hopkins Univ. v. Cellpro, Inc., 152 F.3d 1342, 1361, 47 USPQ2d 1705, 1719 (Fed. Cir. 1998); Engel Indus. Inc. v. Lockformer Co., 946 F.2d 1528, 1533, 20 USPQ2d 1300, 1304 (Fed. Cir. 1991)); *see also* Durel Corp. v. Osram Sylvania Inc., 256 F.3d 1298, 1308, 59 USPQ2d 1238, 1244 (Fed. Cir. 2001).

The issue of adequate enablement depends on whether one skilled in the art could reproduce the claimed invention without "undue experimentation." As correctly put forward by the Examiner, enablement by a disclosure is not precluded, even if some experimentation is required, because the only limiting factor is that the experimentation must not be "undue." In re Wands, 858 F.2d 731, 8 USPQ2d 1400 (Fed. Cir. 1988). In Wands, Judge Smith decided that the key word in this formula is "undue" not "experimentation" and applied a reasonableness standard, given the nature of the invention and the state of the art, when he stated:

> *"The test is not merely quantitative, since a considerable amount of experimentation is permissible, if it is merely routine, or if the specification in question provides a reasonable amount of*

> *guidance with respect to the direction in which the experimentation should proceed."* Wands at 737.

The point of the above quote is that, when the Applicant, as in the instant specification, provide a working example, give extensive guidance to appropriate protocols through the specification --referenced by citation to specific literature, and incorporate in the invention many techniques that are extremely well known and routine in the art, any experimentation that may be necessary, becomes routine. The practitioner in the field knows that the methodologies described in the instant specification will work.

The extensive protocols disclosed in the specification, provide the public the ability to practice the invention, essentially by providing a detailed map. In conjunction with the extremely high level of skill in the field, it is clear that the specification, as tempered by the relevant case law discussed above, does enable other workers in the field to make and use the invention without "excessive" or "undue" experimentation. Wands at 740.

The Examiner states "the specification is not enabling for the claimed invention because the art of gene therapy, in particular the delivery of a DNA vaccine which encodes undefined immunogenic epitopes, is highly unpredictable as recognized in the prior art." The applicant respectfully disagrees with this statement as the science and techniques of immunology are well characterized and predictable to those skilled in the art.

The Examiner's position is in conflict with published Patent Office Guidelines related to written description expressed in the document entitled "Revised Interim Written Description Guidelines Training Materials" and available at http://www.uspto.gov/web/menu/written.pdf. The closest example in that publication is found Example 16: Antibodies wherein:

> *"[The] specification provides a clear protocol by which antigen X was isolated. The specification contemplates but does not teach in an example antibodies which specifically bind to antigen X."*

With regard to a claim to an antibody capable of binding antigen X, the guidelines state the following.

> *"Considering the routine art-recognized method of making antibodies to fully characterized antigens, the well defined structural characteristics for the five classes of antibody, the functional characteristics of antibody binding, and the fact that the antibody technology is well developed and mature, one of skill in the art would have recognized that the spectrum of antibodies which bind to antigen X were implicitly disclosed as a result of the isolation of antigen X."*

There are certainly differences between the claimed invention and this example, and the training materials relate to written description vs. enablement, however, the referenced sections still serve as an articulation of the PTO's prevailing view of the state of immunology today.

Referring specifically to claims 1 and 2, recombinant DNA is claimed. The genomic fragment was cloned using antisera from animals which were previously infected with *E. canis*, and the open reading frames in the genomic fragment were deduced and recited in the specification and sequence listing and recited in claim 1 subparagraphs (a) - (e). These are clearly enabled by the specification. The Examiner's concern seems to focus on claim 1, subparagraph (f) which recites DNA fragments that encode proteins that elicit an immune response against *E. canis*. The specification clearly teaches how to determine and identify DNA that encodes such proteins in the very selection process for the initial isolated fragments. It is a matter of routine experimentation to produce recombinant fragments in expression vectors and to see whether they bind to antibodies against *E. canis*.

Throughout the Office Action, the Examiner takes issue with the fact that various results were not demonstrated. However, there is no requirement for actual reduction to practice or provision of "prototypes" under the current patent law. It is not necessary for the applicant to show binding for every possible recombination, or even any. The parameters are clearly recited in the claim and the techniques for creating a recombinant DNA that meets those parameters described in specification. No undue experimentation is required. As such

claims 1 and 2 are sufficiently enabled. Reconsideration and withdrawal of the rejection is requested.

With regard to claims 6-11, the specification teaches a vaccine that is a recombinant vector that expresses one of the disclosed fragments in a host thereby generating an immune response. The support for theses claims is found throughout the application, but specific examples are provided on pages 7-8.

The specification teaches that the specifically recited sequences and fragments that encode proteins that generate an immune response can be used in a DNA vaccine. As stated above, the methodology of determining which sequences generate an immune response is described in this section of the application and as part of the initial screening. As such the specific selection of the sequences covered by the recitation of subparagraph b) of claim 6 and subparagraph a) of claim 26 are enabled. Further, promoter selection and verification of expression is described, such that creation of the vector as recited in the first part of subparagraph a) of claim 6 and subparagraph b) of claim 26 are both enabled. Lastly, the delivery of the vector is taught such that the last half of subparagraph a) of claim 6 is enabled.

On these issues the Examiner's seems to agree:

> "*the specification demonstrates that antisera from dogs can react with a protein which is encoded by the genomic fragment...*"
>
> "*the proposed open reading frames in the proper context can encode a protein and there are examples in the art that demonstrate that one can produce an immune response when one injects enough of almost any protein....*"

Not to mischaracterize the Examiner's comments, in each case the Examiner goes on to state various concerns related to the failure of the application to "demonstrate" various scientific facts that are not recited in the claim. However, the failure to provide experiments that might be desirable for academic publication or thesis work is not prohibitive of allowance of the claims. The steps of making and using the materials recited in the claim are clearly taught in the specification and that is all that is required.

To further support the Applicant's position that the specification indeed enables one skilled in the art to make and/or use the claimed invention recited in claims 1, 2, 5-11, 26-31 and 47-50, the Applicant submits herewith a Declaration of Yung-Fu Chang, Ph.D. Under 37 C.F.R. § 1.132. In the Declaration, Dr. Chang declares that as of the earliest priority date of the claimed invention, a practitioner in the field could have used the teachings of the specification, coupled with routine methods and knowledge in the art, to practice the claimed invention without undue experimentation.

In addition, claims 9 and 29 have been rejected under 35 U.S.C. §112, first paragraph, as containing non-enabled subject matter because, as noted by the Examiner at page 12 of the Office Action, "the specification does not provide any specific sequence information for any of the four claimed vectors," i.e., vectors pcDNA3, pC1, VR1012, and VR1020. The vectors pcDNA3, pC1, VR1012, and VR1020 are well-known in the art and are commercially available. pcDNA3 is commercially available from, e.g., Invitrogen, Carlsbad, CA (see listing in Invitrogen on-line catalog, Exhibit 1). pCI is commercially available from, e.g., Promega Corp., Madison, WI (see listing in Promega on-line catalog, Exhibit 2). VR1012 and VR1020 are both commercially available from e.g., Vical, San Diego, CA (see, e.g., Wizel et al., 1998, Infect. Immun. 66 (11): 5073–5081 at page 5074, "Plasmid DNA constructs", Exhibit 3). Since the above-described vectors are well-known in the art and are commercially available, it is respectfully requested that the rejection of claims 9 and 29 under 35 U.S.C. §112, first paragraph, be reconsidered and withdrawn.

It is respectfully submitted that the rejection of claims 1, 2, 5-11, 26-31 and 47-50 under 35 U.S.C. §112, first paragraph, is thus overcome. Reconsideration and withdrawal of the rejection of claims 1, 2, 5-11 and 26-31 and 47-50 as lacking an enabling disclosure are therefore respectfully requested.

b. <u>Examiner's Rejections Under 35 U.S.C. § 102(b) Should Be Withdrawn</u>

Claims 1-2 and 47-50 were rejected under 35 U.S.C. 102(b), as being anticipated by Lewis *et al.* (Sequence, organization, and evolution of the A+T region of *Drosophila*

melanogaster mitochondrial DNA. Mol. Biol. Evol. 11: 523-538). The Applicant respectfully disagrees with the rejection.

Claim 1 recites a recombinant DNA that encodes a protein having an amino acid sequence as shown in SEQ. ID. NOS. 3, 5, 7, 9 or 11, or any portion of said DNA above that encodes a protein that elicits an immune response against *E. canis*.

Claim 47 recites a recombinant DNA that encodes a protein having an amino acid sequence as shown in SEQ. ID. NOS. 3, 5, 7, 9 or 11.

Claim 48 recites a vector capable of expressing a recombinant DNA comprising a recombinant DNA inserted into said vector such that a recombinant protein is expressed when said vector is provided in an appropriate host wherein said DNA is selected from the group consisting of a recombinant DNA sequence that encodes a protein having an amino acid sequence as shown in SEQ. ID. NOS. 3, 5, 7, 9 or 11; and any portion of said DNA above that encodes a protein that elicits an immune response against *E. canis*.

Claim 50 recites a vector capable of expressing a recombinant DNA comprising a recombinant DNA inserted into said vector such that a recombinant protein is expressed when said vector is provided in an appropriate host wherein said DNA is selected from the group consisting of a recombinant DNA that encodes a protein having an amino acid sequence as shown in SEQ. ID. NOS. 3, 5, 7, 9 or 11.

Lewis *et al.* does not disclose any recombinant DNA that encodes a protein having an amino acid sequence as shown in SEQ. ID. NOS. 3, 5, 7, 9 or 11. Further, Lewis *et al.* does not disclose a portion of any recombinant DNA that encodes a protein that elicits an immune response against *E. canis*. Rather, Lewis *et al.* merely discloses the DNA sequence of the long (4.6-kb) A+T region of *Drosophila melanogaster* mitochondrial DNA. There is no disclosure of any recombinant DNA that encodes a protein that elicits an immune response against *E. canis;* indeed, the reference is entirely silent regarding *E. canis* and the immune response generally. There is no disclosure of any of the claimed sequences, and no teaching

that any of the DNAs disclosed in the reference encode a protein that elicits an immune response in any animal against *E. canis*.

Furthermore, if the Examiner relies upon the theory of inherency, then the Examiner must provide a basis in fact and/or technical reasoning to reasonably support the determination that the allegedly inherent characteristic necessarily flows from the teachings of the applied prior art. Ex parte Levy, 17 USPQ2d 1461, 1464 (Bd. Pat. App. & Inter. 1990). The fact that a certain result or characteristic may occur or be present in the prior art is not sufficient to establish the inherency of that result or characteristic. In re Rijckaert, 9 F.3d 1531, 1534, 28 USPQ2d 1955, 1957 (Fed. Cir. 1993); In re Oelrich, 666 F.2d 578, 581-82, 212 USPQ 323, 326 (CCPA 1981). It is noted that the Examiner has not provided any reasonable basis in fact and/or technical reasoning to support a rejection for anticipation by inherency.

Since the reference does not disclose each and every element of claims 1, 47, 48, and 50, Lewis *et al.* cannot anticipate the claims. Claim 2, being dependent upon and further limiting claim 1, and claim 49, being dependent upon and further limiting claim 47, should also be allowable for that reason, as well as for the additional limitations recited therein.

It is respectfully submitted that the rejection is thus overcome. Reconsideration and withdrawal of the rejection of claims 1-2 and 47-50 as being anticipated by Lewis *et al.* are therefore respectfully requested.

CONCLUSION

In view of the foregoing amendments and remarks, the Applicant believes that the application is in good and proper condition for allowance. Early notification to that effect is earnestly solicited. If the Examiner feels that a telephone call would expedite the consideration of the application, the Examiner is invited to call the undersigned attorney at (315) 425-9000.

If there are any other fees due in connection with the filing of this Amendment or accompanying papers, please charge the fees to Wall Marjama and Bilinski LLP's Deposit Account No. **50-0289**. If a fee is required for an extension of time under 37 C.F.R. § 1.136 not accounted for above, such an extension is requested and the fee should also be charged to the Deposit Account.

Respectfully submitted,

Date: July 22, 2005

Anne M Schneiderman 43,095
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Syracuse, NY 13202
(315) 425-9000

Enclosures

Appl. No. 10/004,494
Amdt. dated July 22, 2005
Reply to Office Action of January 24, 2005
Exhibit 1

pcDNA™3.1 Directional TOPO® Expression Kit

** Your account specific pricing will be displayed once an item has been added to your order.*

Product	Tech. Docs.	Cat. No.	Size	List Price*	Qty
pcDNA™3.1 Directional TOPO® Expression Kit		K4900-01	20 rxns	419.00	
pcDNA™3.1 Directional TOPO® Expression Kit		K4900-40	40 rxns	814.00	

For additional product info, click the associated Tech. Docs. icon.

Add To Favorites Add To Order

Product Image(s)

Figure 1

Fast, Directional Cloning and High-Level Expression from the CMV Promoter

Description:
The pcDNA™3.1 Directional TOPO® Expression Kit uses linearized, topoisomerase I-activated pcDNA3.1D/V5-His-TOPO® for five-minute directional cloning and subsequent high-level expression. Directional Cloning technology facilitates expression experiments because:

- A proofreading enzyme is used for fewer errors in cloned genes
- Greater than 90% of the clones are in the correct orientation for expression, reducing time spent colony screening for clone orientation

In addition, pcDNA3.1D/V5-His-TOPO¤ provides strong expression levels from the CMV promoter and the option of a C-terminal V5-His fusion tag for easy detection of recombinant protein with an Anti-V5 Antibody and rapid purification on nickel-chelating resin.

Contents and Storage:
Each pcDNA™3.1 Directional TOPO¤ Expression Kit contains two boxes. The TOPO® box contains linearized and topoisomerase I-activated pcDNA3.1D/V5-His-TOPO® vector, dNTPs, control template and primers, primers for sequencing, and an expression control plasmid. Store at -20°C. The One Shot® box contains single-use 50-µl aliquots of One Shot® TOP10 Chemically Competent *E. coli*, S.O.C. medium, and pUC19 supercoiled plasmid control. Store at -80°C. Guaranteed stable for 6 months when properly stored.

Rela
Anti
Anti
Gen
Sele
Anti
Lipo
200
Trar
Rea
Ni-N
Puri
Syst
Pr
Su
Cust
Sup

Appl. No. 10/004,494
Amdt. dated July 22, 2005
Reply to Office Action of January 24, 2005
Exhibit 2

Promega

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pCI Mammalian Expression Vector

Back to Product Groups

Product	Cat.#	Size	Qty.	Price	Order Q
pCI Mammalian Expression Vector	E1731	20µg	Each	$ 213.00	1

Component Listing View Figures

Description

The pCI Mammalian Expression Vector[a] promotes constitutive ex cloned DNA inserts in mammalian cells. The major difference betw pCI and pSI Mammalian Expression Vectors is the enhancer/prom controlling the expression of the inserted gene. The pCI Expressio contains the human cytomegalovirus (CMV) major immediate-earl enhancer/promoter region. This vector can be used for both transi stable expression of genes. For stable expression, the pCI Vector r co-transfected with an expression vector containing a selectable g mammalian cells.

Features

- **Strong, Constitutive Expression:** The pCI Vector's CMV enhancer/promoter region enables strong, constitutive exp many cell types. A β-globin/IgG chimeric intron located dov of the enhancer/promoter region can further increase expr 3).
- **Increased Steady-State mRNA Levels:** The late SV40 polyadenylation signal increases the steady-state level of R approximately fivefold more than the early SV40 polyaden signal (4).
- **Convenient:** Multiple cloning sites exist for easy insertion
- **Versatile:** Synthesize transcripts in vitro using the T7 RNA polymerase promoter or generate single-stranded DNA in *E* the f1 origin of replication.

Protocol Technical Bulletin #TB206.

Vector Sequence pCI Mammalian Expression Vector sequence.

Storage Conditions Store at –20°C.

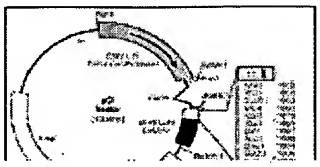
GenBank®/EMBL Accession Number U47119.

References

1. Brinster, R.L. *et al.* (1988) *Proc. Natl. Acad. Sci. USA* **85**, 8
2. Choi, T. *et al.* (1991) *Mol. Cell. Biol.* **11**, 3070–4.
3. Palmiter, R.D. *et al.* (1991) *Proc. Natl. Acad. Sci. USA* **88**,
4. Carswell, S. and Alwine, J.C. (1989) *Mol. Cell. Biol.* **9**, 424

Patents/Disclaimers [a]The CMV promoter and its use are covered under U.S. Pat. Nos. and 5,385,839 owned by the University of Iowa Research Foundat City, Iowa, and licensed FOR RESEARCH USE ONLY. Commercial u obtain a license to these patents directly from the University of Io Research Foundation.

Figures (click image to enlarge)



Cat.# E1731.

Additional Information

PN049 *Promega Notes* 49

Related Products

pCI-neo Mammalian Expression Vector
pSI Mammalian Expression Vector
pAdVAntage™ Vector
T7 EEV Promoter Primer
ProFection® Mammalian Transfection System—Calcium Phos
TransFast™ Transfection Reagent
Transfectam® Reagent for the Transfection of Eukaryotic Ce
PureYield™ Plasmid Midiprep System

INFECTION AND IMMUNITY, Nov. 1998, p. 5073–5081 Vol. 66, No. 11
0019-9567/98/$04.00+0

Vaccination with Trypomastigote Surface Antigen 1-Encoding Plasmid DNA Confers Protection against Lethal *Trypanosoma cruzi* Infection

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Received 7 May 1998/Returned for modification 23 July 1998/Accepted 10 August 1998

DNA vaccination was evaluated with the experimental murine model of *Trypanosoma cruzi* infection as a means to induce antiparasite protective immunity, and the trypomastigote surface antigen 1 (TSA-1), a target of anti-*T. cruzi* antibody and major histocompatibility complex (MHC) class I-restricted CD8$^+$ cytotoxic T-lymphocyte (CTL) responses, was used as the model antigen. Following the intramuscular immunization of *H-2^b* and *H-2^d* mice with a plasmid DNA encoding an N-terminally truncated TSA-1 lacking or containing the C-terminal nonapeptide tandem repeats, the antibody level, CTL response, and protection against challenge with *T. cruzi* were assessed. In *H-2^b* mice, antiparasite antibodies were induced only by immunization with the DNA construct encoding TSA-1 containing the C-terminal repeats. However, both DNA constructs were efficient in eliciting long-lasting CTL responses against the protective *H-2K^b*-restricted TSA-1$_{515–522}$ epitope. In *H-2^d* mice, inoculation with either of the two TSA-1-expressing vectors effectively generated antiparasite antibodies and primed CTLs that lysed *T. cruzi*-infected cells in an antigen-specific, MHC class I-restricted, and CD8$^+$-T-cell-dependent manner. When TSA-1 DNA-vaccinated animals were challenged with *T. cruzi*, 14 of 22 (64%) *H-2^b* and 16 of 18 (89%) *H-2^d* mice survived the infection. The ability to induce significant murine anti-*T. cruzi* protective immunity by immunization with plasmid DNA expressing TSA-1 provides the basis for the application of this technology in the design of optimal DNA multicomponent anti-*T. cruzi* vaccines which may ultimately be used for the prevention or treatment of Chagas' disease.

Chagas' disease, caused by the intracellular protozoan parasite *Trypanosoma cruzi*, is a lifelong health problem in Central and South America, where an estimated 18 million people are infected with this parasite and 90 million are at risk of infection (35, 65). Following a short-lived acute-phase illness characterized by fever and a patent parasitemia, infected individuals enter a nearly aparasitemic asymptomatic chronic phase, where most remain for the remainder of their lifetime. However, at 10 to 20 years postinfection nearly 30% of infected individuals develop severe cardiomyopathy, which is responsible for most of the 50,000 deaths caused by Chagas' disease each year (45). Although reduviid vector control and blood bank screening measures have had a major impact in reducing transmission of *T. cruzi* (65), the operational costs to maintain such control programs, behavioral differences among vector species, existence of animal reservoirs, persistence of parasites in chronically infected patients, and lack of adequate chemotherapies to treat the infection will likely prevent these control measures alone from completely eradicating *T. cruzi*. An additional approach that could contribute significantly to control the transmission of Chagas' disease is the development of anti-*T. cruzi* vaccines. To date, however, vaccine production for *T. cruzi* has been a low priority despite the current knowledge about the protective roles that antibodies, type 1 cytokines, and CD8$^+$ T cells play in resistance to experimental *T. cruzi* infections (53).

During *T. cruzi* infection, both chagasic patients and experimental animals produce strong immune responses to molecules from the infective nonreplicative trypomastigote stage and the replicative amastigote forms (3, 4, 14, 29). Among these, trypomastigote surface antigen 1 (TSA-1) (15, 38), a major trypomastigote surface antigen and the first identified member of the *trans*-sialidase gene superfamily (48), is a target of protective immune responses in mice (61, 66). Immunization with an amino-proximal fragment of TSA-1 induces a strong antibody response and protects mice against an otherwise lethal challenge with *T. cruzi* (66). Our studies have recently identified TSA-1 as the first bona fide target of CD8$^+$ cytotoxic T lymphocytes (CTL) in *T. cruzi*-infected mice and demonstrated that the adoptive transfer of TSA-1-specific gamma interferon (IFN-γ)- and tumor necrosis factor alpha-producing CTL lines protects naive animals against lethal *T. cruzi* infection (61). Moreover, we have recently determined that TSA-1 and amastigote surface protein-1 and -2 (33, 44), which are also recognized by murine CTL (32), represent three target molecules of *T. cruzi*-specific human CD8$^+$ CTL (62). These studies demonstrated the validity of the mouse model to identify target antigens of protective anti-*T. cruzi* immune responses and provide a strong incentive for the development of vaccines as a potential control measure against Chagas' disease. For this purpose, and given the success of plasmid DNA vaccination in specifically stimulating a broad spectrum of immune responses to the vector-encoded target antigen (12), we have chosen to investigate DNA-based immunization as a system to generate vaccine-induced resistance against *T. cruzi* and have used TSA-1 as a model antigen for its initial evaluation. In this report we document that intramuscular injection of BALB/c and C57BL/6J mice with TSA-1-encoding plasmid DNA induces antibodies, CTL, and significant protection against lethal challenge with *T. cruzi*.

MATERIALS AND METHODS

Mice and parasites. Six- to 8-week-old female C57BL/6J (B6) and BALB/cByJ (BALB/c) mice (breeding pairs obtained from The Jackson Laboratory, Bar Harbor, Maine) were used in all experiments. The Brazil strain of *T. cruzi* was maintained in vivo by serial biweekly passage of 10^3 blood-form trypomastigotes (BFT) in C3H/HeSnJ mice (30) and by continuous in vitro passages of tissue culture-derived trypomastigotes (TCT) in monolayers of Vero cells (18). B6 mice

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were infected intraperitoneally with 10^3 BFT and challenged 3 months later with 10^5 TCT by subcutaneous injection at the base of the tail.

Cell lines and culture reagents. P815 cells (*H-2*^{d}; mastocytoma cells; ATCC TIB 64), J774 cells (*H-2*^{d}; macrophages; ATCC TIB 67), 3T3 cells (*H-2*^{d}; fibroblasts; ATCC CCL 163), and Vero cells (African Green monkey kidney cells; ATCC CCL 81) (all from the American Type Culture Collection, Rockville, Md.); RMA-S cells (peptide TAP.2 transporter-deficient, low *H-2*^{b} expressor mutant of the RBL-5 Rauscher virus-induced T-cell lymphoma; provided by H.-G. Ljundggren, Karolinska Institute, Stockholm, Sweden); and 5A.Kb.α3 cells (*H-2*^{k} fibroblasts stably transfected with the K^b gene; provided by S. Jameson, University of Minnesotta, Minneapolis) were maintained in complete RPMI 1640 (Mediatech, Herndon, Va.) medium (CR) containing 10% heat-inactivated fetal bovine serum (HyClone, Logan, Utah), 20 mM HEPES, 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids and 50 μg of gentamicin per ml (all from Gibco BRL, Gaithersburg, Md.). COS-7 cells (simian virus 40-transformed African Green monkey kidney cells; ATCC CRL 1651) were grown in similarly supplemented Dulbecco's modified Eagle's medium (DMEM) (Mediatech). T-cell medium (TCM) was prepared by supplementing CR with 50 μM 2-mercaptoethanol (Gibco BRL).

Peptides. The peptide TSA-$1_{515-522}$ (VDYNFTIV) (61), representing the *H-2K*^{b}-restricted *T. cruzi* TSA-1 CTL epitope, was produced by using 9-fluorenylmethoxycarbonyl-based solid-phase chemistry on an ACT MPS 350 peptide synthesizer (Advanced Chem Tech, Louisville, Ky.) by the Molecular and Genetic Instrumentation Facility at the University of Georgia (Athens). The *H-2K*^{b}-restricted OVA CTL peptide $OVA_{257-264}$ (SIINFEKL) was used as a control (42). Lyophilized peptides were dissolved at 20 mg/ml in dimethyl sulfoxide and stored at −70°C. Before use, peptides were diluted with RPMI 1640. Peptides were not toxic to target cells or effector cell cultures.

Plasmid DNA constructs. The genomic DNA fragments of the TSA-1 gene (15, 38) encoding amino acid residues 78 to 652 and 78 to 790, excluding and including, respectively, the (5)-nonapeptide tandem repeat unit, were amplified by PCR with pBluescript II SK (+)/TSA-1 (provided by David Fouts, University of California, Irvine) as a template. Forward and reverse primers were designed to incorporate, respectively, *Sal*I and *Xba*I restriction sites (underlined below) for directional cloning. Primers were constructed on an Applied Biosystems (Foster City, Calif.) 394 DNA/RNA synthesizer at the Molecular Genetics Instrumentation Facility. The forward oligonucleotide primer 5′-AGTCGACGG ATCCATGATTGCATTTGTCGAAGGC-3′ was used with reverse primers 5′-ATCTAGAAGCTTCATAGTTCACCGACACTCAGTGG-3′ and 5′-ATCTAGAAGCTTCATGCCGCAGCATTTGCTTCCCC-3′ to amplify a 1.7-kb (repeatless TSA-1_{78-652}) and a 2.1-kb (repeat-bearing TSA-1_{78-790}) product, respectively. The amplification products containing the A overhangs generated by *Taq* DNA polymerase during the PCR were cloned into the *Hinc*II site of the pUC19-*T* vector. Following digestion with *Sal*I and *Xba*I, the 1.7- and 2.1-kb TSA-1 fragments were gel purified and cloned into the *Sal*I and *Xba*I sites of the eukaryotic expression vector VR1012 (Vical Inc., San Diego, Calif.) (19) to generate VR1012 TSA1.7 and VR1012 TSA2.1. In the VR1012 vector, expression of the encoded gene is driven by a cytomegalovirus immediate-early gene promoter. Constructs were transformed into *Escherichia coli* DH5α competent cells and grown in Luria-Bertani broth with 70 μg of kanamycin per ml as described previously (43). Closed circular plasmid DNA was purified by anion-exchange chromatography with the Qiagen (Chatsworth, Calif.) maxi prep kit according to the manufacturer's specifications. Plasmid DNA was sterilized by ethanol precipitation and dissolved in sterile phosphate-buffered saline (PBS).

In vitro expression. Expression of VR1012 TSA1.7 and VR1012 TSA2.1 in COS-7 cells was assessed in vitro by transient transfection. COS-7 cells were seeded in six-well plates (Costar, Cambridge, Mass.) at 2×10^5 cells/well in 3 ml of complete DMEM and incubated overnight at 37°C and 6% CO_2. In a final volume of 300 μl, 10 μg of plasmid DNA was mixed with 30 μg of Lipofectin reagent (Gibco BRL), and the mixture was incubated for 15 min at room temperature before being diluted with 1.7 ml of serum-free MEM. After the COS-7 monolayers (50 to 70% confluent) were washed with serum-free MEM, cells were overlaid with the mixture containing the DNA-Lipofectin complexes and incubated overnight at 37°C and 6% CO_2. The cell culture medium was then replaced with 3 ml of complete DMEM and incubated for an additional day. Transiently transfected COS-7 cells were harvested by gentle trypsinization, washed in PBS, and seeded in eight-well Lab Tek chamber slides (Nunc Inc., Naperville, Ill.) at 10^4 cells/well. After overnight incubation at 37°C and 6% CO_2, cells were washed with PBS, fixed in ice-cold methanol for 15 min at 4°C, and washed four more times before blocking with PBS–1% bovine serum albumin (BSA) for 1 h at 37°C. Cells were subsequently stained for 2 h at 37°C with a polyclonal anti-*T. cruzi* serum obtained from acutely infected C3H/HeSnJ mice or with normal mouse serum (1:200 dilution in PBS–1% BSA), washed three times, and finally incubated for 1 h at room temperature with fluorescein isothiocyanate-labeled $F(ab')_2$ goat anti-mouse immunoglobulin G (1:50 dilution in PBS–1% BSA) (Southern Biotechnology, Birmingham, Ala.). Slides were then rinsed four times with PBS–1% BSA and mounted in 10% glycerol–0.1 M sodium bicarbonate (pH 9)–2.5% 1,4-diazobicyclo[2,2,2]octane for visualization by laser scanning confocal microscopy (MRC-600) (Bio-Rad Laboratories, Hercules, Calif.).

Genetic immunizations and challenges. Groups of B6 and BALB/c mice were injected intramuscularly into each tibialis anterior muscle with 50 μg of VR1012 TSA1.7, VR1012 TSA2.1, or control VR1012 suspended in 50 μl of PBS by using a 27-gauge needle. Mice were boosted 4 weeks later with an identical dose of plasmid (100 μg total) given by the same bilateral intramuscular injection. Tail blood samples were collected 3 and 2 weeks after the first and second doses, respectively, and sera were stored at −20°C until assayed for anti-*T. cruzi* antibody. Two weeks after the second dose, animals were infected by intraperitoneal injection of 10^5 (B6) or 10^3 (BALB/c) *T. cruzi* BFT. Parasitemias were monitored periodically by hemacytometer counts of 10 μl of tail vein blood in an ammonium chloride solution. Mortality was recorded daily.

Determination of serum antibody levels. Antibody responses induced by the immunization of mice with plasmid DNA were evaluated by a solid-phase enzyme-linked immunosorbent assay (ELISA). In brief, capture antigen was prepared by sonication of 5×10^7 PBS-washed *T. cruzi* parasites (80% trypomastigotes, 20% amastigotes) in 50 mM carbonate-bicarbonate buffer (pH 9.6). Sonicated material was spun for 1 h at 100,000 × *g* at 4°C. Wells of flexible polyvinyl chloride 96-well plates (Falcon, Becton Dickinson & Co., Oxnard, Calif.) were coated overnight at 4°C with 100 μl of a predetermined optimal dilution (5×10^5 parasites/well) of the soluble antigen. Washed wells were blocked with 1% BSA in PBS–0.05% Tween 20 (PBST) for 1 h at 37°C. After blocking, 100 μl of pooled mouse sera (1:100 dilution in PBST) was added to the plates and incubated for 1 h at 37°C. Plates were washed six times with PBST and incubated for an additional hour with 100 μl of a horseradish peroxidase-labeled goat anti-mouse immunoglobulin (A, G, M) (1:1,000 dilution in PBST) (Cappel, Organon Teknika Corp., West Chester, Pa.). Washed wells were developed with 100 μl of the substrate 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid), and absorbance was read at 405 nm with an automated ELISA microplate reader (Bio-Tek Instruments, Winooski, Vt.).

Generation of effector cells. Unless otherwise indicated, spleens from DNA-immunized mice were removed 2 weeks after the last dose, and immune spleen cell (SC) suspensions were prepared in TCM. In the case of B6 mice, SCs were cultured in 24-well plates at 5×10^6 cells/well. TSA-$1_{515-522}$ peptide was included in each 2-ml culture at 1 μM (final concentration). In the case of BALB/c mice, 35×10^6 SCs in 10 ml of TCM were cultured in upright 25-cm^2 tissue culture flasks containing irradiated monolayers of stimulator *T. cruzi*-infected J774 cells. After 2 days of incubation at 37°C and 6% CO_2, cultures were made to 5% Rat T-STIM without concanavalin A (Collaborative Biomedical Products, Bedford, Mass.) and incubated for 4 additional days. Effector cells from BALB/c mice were also unstimulated immune SCs without secondary in vitro stimulation. SCs from B6 mice chronically infected with *T. cruzi* were obtained 6 months after parasite challenge and stimulated as described for SCs from DNA-immunized animals.

Preparation of peptide-pulsed target cells. Peptide-pulsed targets were used to measure CTL activity of peptide-stimulated effector cells generated from plasmid DNA-immunized B6 mice. RMA-S (*H-2*^{b}) cells preincubated for 24 h at 26°C and 6% CO_2 were seeded into 24-well plates (Costar) at 10^6 cells/well in 2 ml of CR and incubated overnight under the same conditions in the presence of 0.05 μM TSA-$1_{515-522}$ peptide or $OVA_{257-264}$ negative control peptide and 100 μCi of a sterile $Na_2{}^{51}CrO_4$ solution (^{51}Cr) (Amersham Life Science Corporation, Arlington, Heights, Ill.). Two hours prior to their processing for CTL assays, cells were shifted to 37°C and 6% CO_2. P815 (*H-2*^{d}) target cells were also prepared in 24-well plates by overnight incubation at 37°C and 6% CO_2 with ^{51}Cr and TSA-$1_{515-522}$ peptide.

Preparation of *T. cruzi*-infected stimulator and target cells. *T. cruzi*-infected cells were used to generate and measure the CTL activity of effector cells from plasmid DNA-immunized BALB/c mice. Monolayers of J774 cells (60% confluent) prepared in upright 25-cm^2 tissue culture flasks (Corning, Corning, N.Y.) were infected overnight with *T. cruzi* TCT (50:1 parasite-to-host cell ratio). After extensive washing with serum-free RPMI 1640 to remove noninvading parasites, infected monolayers were irradiated (14 krads) (Gammacell 200; ^{60}Co source) and then used as stimulators for immune SCs. To prepare *T. cruzi*-infected target cells used to ascertain the lytic activity of BALB/c-derived stimulated SCs, monolayers (50% confluent in horizontal 25-cm^2 flasks) of major histocompatibility complex (MHC)-matched 3T3 (*H-2*^{d}) and mismatched 5A.Kb.α3 (*H-2*^{k} and *H-2K*^{b}) cells were incubated for 2 days at 37°C and 6% CO_2 in CR supplemented with 1,000 U of IFN-α plus IFN-β (Lee Biomolecular Laboratories, Inc., San Diego, Calif.) per ml, washed, and then infected overnight with *T. cruzi* TCT (50:1 parasite-to-host cell ratio). After being washed, *T. cruzi*-infected monolayers were treated with PBS–1 mM EDTA to prepare single-cell suspensions and washed once more before a 1-h ^{51}Cr labeling step at 37°C. To assess the lytic activity of unstimulated BALB/c-derived immune SCs, monolayers of untreated J774 cells were infected, and single-cell suspensions for ^{51}Cr labeling were prepared by moderate pipetting of the cell monolayer. Under these conditions, stained (Leukostat; Fisher Scientific, Atlanta, Ga.) cytospin preparations of each culture indicated that 65 to 75% of the cells were infected.

CTL assay. Cytolytic activity was measured by the ^{51}Cr release assay, as previously described (63). In brief, ^{51}Cr-labeled target cells were washed three times in CR and resuspended in TCM, and 5×10^3 target cells (100 μl) were added to effector cells (100 μl) at various effector cell-to-target cell (E/T) ratios in 96-well round-bottom plates (Corning). After a 5-h incubation at 37°C and 6% CO_2, supernatants were harvested with the SCS System (Skatron, Sterling, Va.), and radioactivity was counted on a Cobra II Autogamma counter (Packard Instrument Company, Downers Grove, Ill.). Percent specific lysis was calculated

from the mean of triplicates as 100 × [(experimental release − spontaneous release)/(maximum release − spontaneous release)]. Maximum and spontaneous releases were determined in wells containing no effectors in the presence or absence of 2% Triton X-100, respectively. In experiments where CTL activity of $CD8^+$ and $CD4^+$ T cells was tested, effector cells were depleted by incubation on ice for 30 min with predetermined dilutions of culture supernatants from hybridomas 3.155 (anti-CD8) (ATCC TIB 211) (46) and RL172 (anti-CD4) (8), followed by 30 min at 37°C in the presence of 1:6-diluted rabbit complement (Pel-Freez, Brown Deer, Wis.). Spontaneous release did not exceed 20% of the maximum release. The standard error ranged between 0.02 to 6.1% of the mean.

RESULTS

Expression of TSA-1 in transiently transfected cells. To study the effectiveness of genetic immunization against *T. cruzi*, the TSA-1 gene was subcloned into the VR1012 mammalian expression vector (19), containing the cytomegalovirus promoter and the bovine growth hormone polyadenylation sequences. The constructs VR1012 TSA1.7 and VR1012 TSA2.1 were generated to drive the expression of two N-terminally truncated TSA-1 gene products lacking and bearing, respectively, the five nonapeptide repeats located near the C-terminal end of the TSA-1 protein. Both plasmid constructs expressed the inserted TSA-1 gene fragment upon transient transfection of COS-7 cells. The cytoplasmic expression of TSA-1 in VR1012 TSA1.7- and VR1012 TSA2.1-transfected cells was intense as detected by immunofluorescent staining with a polyclonal anti-*T. cruzi* serum (Fig. 1D and F). In contrast, similarly transfected cells stained with normal mouse serum showed no evidence of immunofluorescence (Fig. 1C and E). No expression was detected in cells transfected with the unmodified VR1012 vector and stained with either serum (Fig. 1A and B).

Immunization with TSA-1 plasmid DNA elicits a parasite-specific antibody response. A strong humoral immune response has been widely implicated as a major effector mechanism that participates in the immune control of *T. cruzi* (28, 29, 71), and immunization of mice with a recombinant N-proximal portion of TSA-1 induces an antibody response which correlates with survival after a lethal challenge infection (66). To ascertain whether a *T. cruzi*-specific antibody response could be elicited by the expression of the TSA-1 protein fragments following intramuscular DNA immunization, BALB/c and B6 mice were injected twice with 100 μg of VR1012 TSA1.7, VR1012 TSA2.1, or control plasmid VR1012. The presence of parasite-specific antibodies in pooled sera prepared from each group of mice was assessed by ELISA (Fig. 2). Three weeks following the first dose, sera from BALB/c mice immunized with either VR1012 TSA1.7 or VR1012 TSA2.1 showed comparable antibody responses against the sonicated parasite material used as capture antigen. Two weeks after the second dose, while a boosting of the parasite-specific antibody level was detected in the sera from the VR1012 TSA1.7-immunized group, the level of antibodies in the sera from the VR1012 TSA2.1-immunized animals remained essentially unchanged. When a similar analysis was conducted for the pooled sera from similarly immunized B6 mice, the antibody levels after the first dose did not exceed the level found in normal mouse serum. However, after the second dose, only the VR1012 TSA2.1-immunized group showed a parasite-specific antibody response. In all cases, the antibody levels detected in the sera from groups of mice immunized with unmodified VR1012 vector were no different than the level measured in normal mouse serum.

Induction of a long-lasting TSA-1-specific CTL response in TSA-1 plasmid DNA-immunized B6 mice. $TSA\text{-}1_{515\text{–}522}$ is a target of $H\text{-}2K^b$-restricted protective CTL responses induced in B6 mice infected with *T. cruzi* (61). We therefore wanted to determine whether immunization of this strain of mice with the TSA-1-encoding DNA vectors could induce a $TSA\text{-}1_{515\text{–}522}$-specific CTL response. Two weeks after the second intramuscular injection of either VR1012 TSA1.7 or VR1012 TSA2.1, immune SCs were stimulated with $TSA\text{-}1_{515\text{–}522}$, and 6 days later, the lytic activity of effectors was tested against peptide-sensitized target cells. CTL activity was antigen specific, MHC class I restricted, and dependent on $CD8^+$ T lymphocytes (Fig. 3A). The $H\text{-}2^b$ effector cells lysed matched RMA-S cells ($H\text{-}2^b$) sensitized with $TSA\text{-}1_{515\text{–}522}$ but were unable to lyse the same cells pulsed with control peptide $OVA_{257\text{–}264}$ or MHC-mismatched P815 cells ($H\text{-}2^d$) pulsed with $TSA\text{-}1_{515\text{–}522}$. Detected lytic activity was abrogated by $CD8^+$-T-cell depletion but not by depletion of $CD4^+$ effectors. In no case did $TSA\text{-}1_{515\text{–}522}$-stimulated SCs from mice immunized with unmodified VR1012 vector display CTL activity against peptide-sensitized target cells. Similar $TSA\text{-}1_{515\text{–}522}$-specific CTL activity was detected in the peptide-stimulated SC cultures established 7 months after mice had received the second 100-μg dose of the TSA-1-encoding DNA vectors (Fig. 3B). The magnitude of such recall CTL responses was comparable to the CTL activity detected for $TSA\text{-}1_{515\text{–}522}$-stimulated effectors from *T. cruzi*-infected mice. Hence, immunization of B6 mice with both TSA-1-encoding DNA constructs generates a long-lasting $TSA\text{-}1_{515\text{–}522}$-specific CTL response which closely resembles the recall response induced in *T. cruzi*-infected animals.

The CTL response induced in BALB/c mice by TSA-1 plasmid DNA immunization is parasite specific, MHC class I restricted, and $CD8^+$ T cell dependent. Despite the fact that the target antigens recognized by CTL from *T. cruzi*-infected BALB/c mice ($H\text{-}2^d$) have not been identified, SCs from these animals display genetically restricted CTL activity against *T. cruzi*-infected target cells (36). Thus, we used this system to determine whether parasite-specific CTL could be induced in BALB/c mice following immunization with the TSA-1-encoding plasmid DNA constructs. First, spleens were collected 2 weeks after the second dose of DNA, and on the same day the CTL activity of SCs against infected and uninfected target cells was measured (Fig. 4A). Infection of J774 cells ($H\text{-}2^d$) with *T. cruzi* efficiently targeted these macrophages for lysis by the $H\text{-}2^d$ effector cells harvested from either VR1012 TSA1.7- or VR1012 TSA2.1-immunized mice. In contrast, minimal or no lysis against uninfected J774 cells and against mismatched *T. cruzi*-infected 5A.Kb.α3 fibroblasts ($H\text{-}2^k$; $H\text{-}2K^b$) was detected. None of the target cells tested was recognized by effector cells obtained from control VR1012-immunized animals. Next, CTL activity of immune SCs that had been stimulated for 6 days with *T. cruzi*-infected J774 macrophages against uninfected and *T. cruzi*-infected fibroblasts was assessed (Fig. 4B). Again, the specificity and MHC class I-restricted nature of the recall CTL response was demonstrated by the ability of effector cells derived from VR1012 TSA1.7- and VR1012 TSA2.1-immunized mice to lyse infected but not uninfected 3T3 cells ($H\text{-}2^d$) and by their inability to recognize infected 5A.Kb.α3 cells ($H\text{-}2^k$; $H\text{-}2K^b$). When the phenotype of the VR1012 TSA1.7-derived effectors was tested, it was found that they were $CD8^+$ $CD4^-$, because the lytic activity of these cells was significantly reduced by the depletion of $CD8^+$ T cells and was minimally affected by the depletion of $CD4^+$ T cells. Similarly stimulated VR1012 immune SCs failed to lyse all the target cells tested. Together, these data indicated that immunization of BALB/c mice with TSA-1-encoding DNA plasmids efficiently primed parasite-specific $CD8^+$ CTL precursors and that these in vivo-expanded cells were in sufficient numbers to allow the detection of their genetically restricted lytic activity without in vitro restimulation.

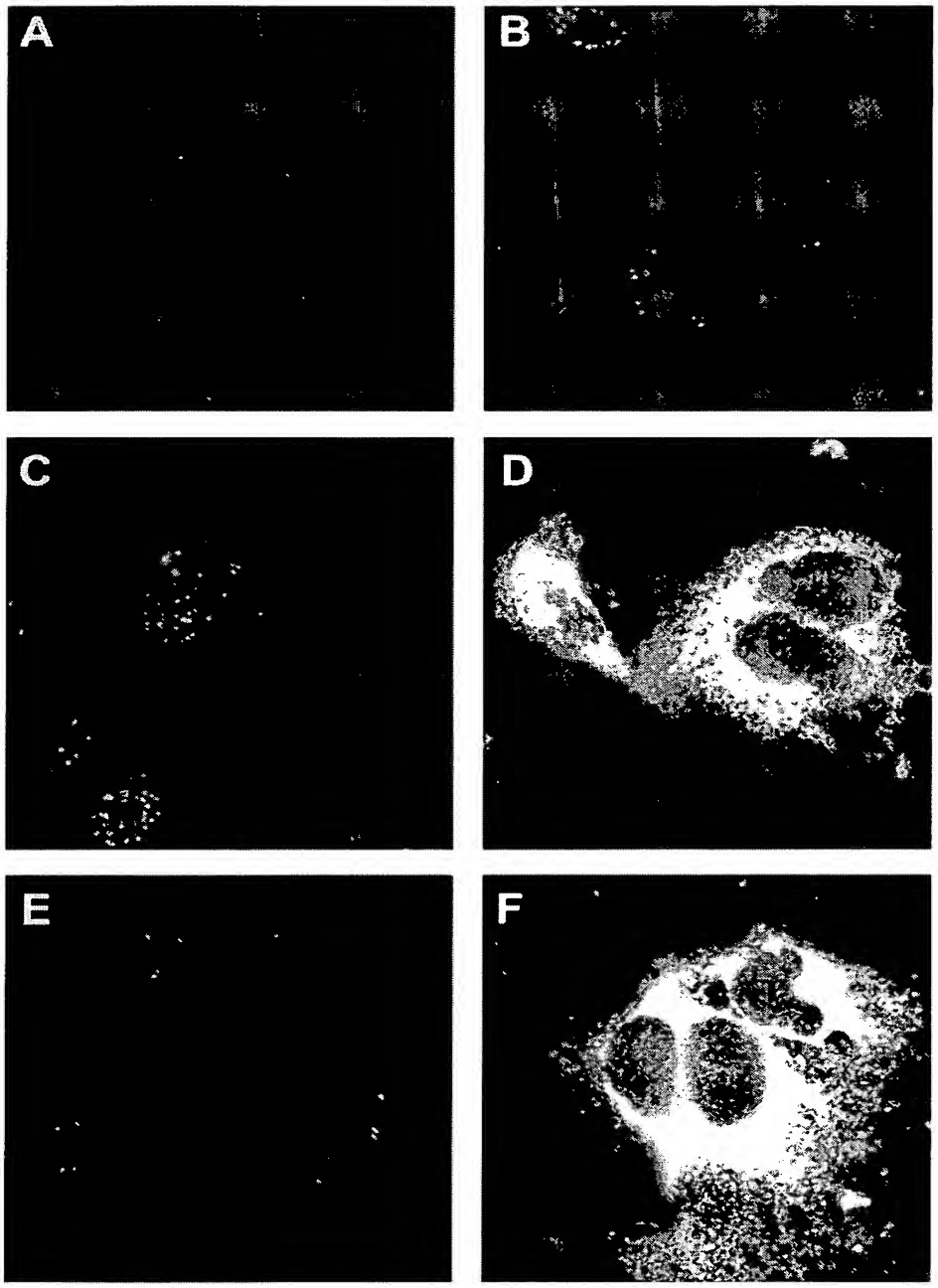

FIG. 1. Expression of TSA-1 in transiently transfected cells. COS-7 cells were transfected with 10 μg of unmodified VR1012 plasmid (A and B), TSA-1-encoding VR1012 TSA1.7 (C and D), or VR1012 TSA2.1 (E and F) by using Lipofection. After 72 h, cells were fixed in ice-cold methanol and stained by immunofluorescence with a polyclonal anti-*T. cruzi* serum obtained from acutely infected mice (B, D, and F) or with a control normal mouse serum (A, C, and E) followed by a fluorescein isothiocyanate-conjugated secondary antibody. Photomicrographs (magnification, ×100) were taken by confocal microscopy. Note the cytoplasmic localization of the transgene-expressed TSA-1 products (D and F).

A TSA-1 plasmid DNA-based vaccine significantly protects mice from *T. cruzi*-induced mortality. Having established that B6 and BALB/c mice generated *T. cruzi*-specific immune responses upon immunization with either of the TSA-1-expressing constructs, we next determined whether DNA vaccination could provide these animals with any degree of protection against challenge with *T. cruzi*. Two weeks after the second immunizing dose, groups of B6 and BALB/c mice were challenged with 10^5 or 10^3 *T. cruzi* BFT, respectively. The difference in the challenging dose was to compensate for the ob-

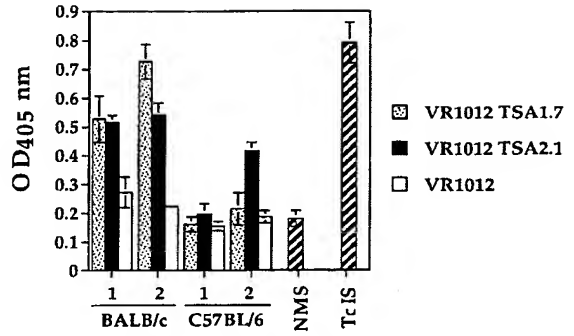

FIG. 2. *T. cruzi*-specific serum antibody response in TSA-1 DNA-vaccinated mice. BALB/c and B6 mice were injected with 50 μg of VR1012 TSA1.7, VR1012 TSA2.1, or unmodified VR1012 plasmid in each tibialis anterior muscle. Mice were boosted after 4 weeks with the same dose of plasmid. The presence of parasite-specific antibodies was assessed by ELISA with a 1:100 dilution of sera pooled from individual tail blood samples (four or five mice per group) and collected 3 and 2 weeks after the first (bars 1) and second (bars 2) doses. Negative and positive controls were sera from normal mice (NMS) and from mice acutely infected with *T. cruzi* (TcIS). OD_{450}, optical density at 450 nm.

served differences in susceptibility of the two strains of mice. Both strains of mice showed a significant degree of protection against *T. cruzi*-induced mortality. As illustrated in one of three conducted experiments, B6 mice vaccinated with either of the TSA-1-encoding vectors showed a 7-day delay in the onset of parasitemia and a consistently reduced level of parasites compared to control animals immunized with the unmodified VR1012 vector (Fig. 5A). Moreover, all control animals died before 45 days postinfection, whereas 50% of mice in each of the test groups survived the infection (Fig. 5B). In the case of BALB/c mice, however, the steady increase in parasitemia noted in TSA-1 DNA-vaccinated animals was strikingly similar to the kinetics of infection observed for mice immunized with the unmodified plasmid DNA (Fig. 6A). Despite similar levels of circulating parasites in test and control animals, none of the mice vaccinated with either of the TSA-1-encoding vectors succumbed to *T. cruzi* infection, whereas 75% of control mice developed fatal infections within 27 days postinfection (Fig. 6B). Overall, protection against an otherwise lethal inoculum with trypomastigotes was observed in 73 and 55% of VR1012 TSA1.7- and VR1012 TSA2.1-vaccinated B6 mice, respectively, and in 91 and 86% of similarly vaccinated BALB/c mice, respectively (Table 1). In contrast, control VR1012-vaccinated mice remained highly susceptible to *T. cruzi*-induced lethality, as only 9% overall survival was observed for both strains (Table 1).

DISCUSSION

Several observations on *T. cruzi*-infected hosts regarding the mechanisms involved in disease development and protective immunity provide strong support for the development of vaccines as a means to prevent or lessen the severity of Chagas' disease (20–22, 41, 53, 56). Thus far, the exploration of vaccines against *T. cruzi* has been widely avoided due to the fear that such intervention methods would exacerbate rather than prevent a disease that many still consider to have an autoimmune etiology (26). However, a growing body of evidence indicates that it is the persistence of *T. cruzi* in the diseased tissue and not the parasite-induced immune responses to self molecules which correlates best with the induction and maintenance of the inflammatory disease process (5, 7, 25, 31, 56).

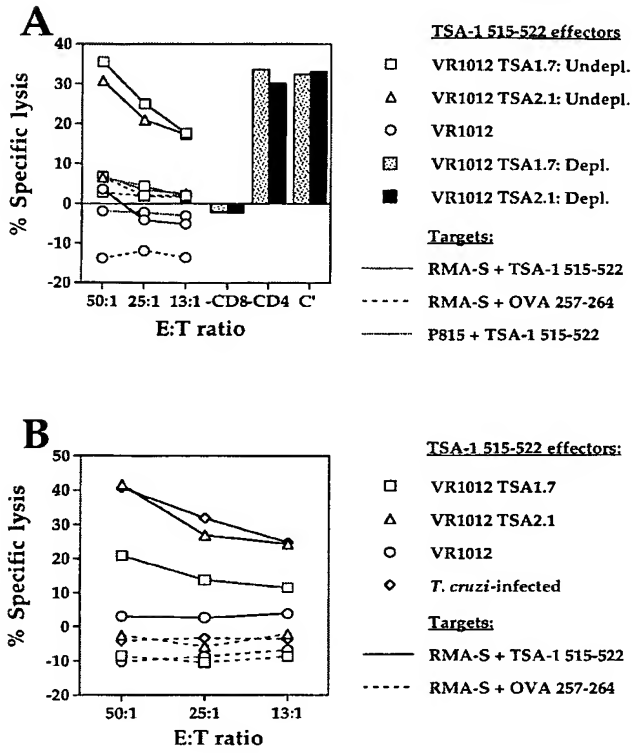

FIG. 3. Induction of long-lasting TSA-$1_{515-522}$-specific, $CD8^+$-T-cell-dependent, MHC class I-restricted CTL in TSA-1 DNA-immunized B6 mice. Four weeks following the first 100-μg intramuscular dose of plasmid DNA, B6 ($H\text{-}2^b$) mice were injected via the same route with an identical dose of the priming vaccine, consisting of either control VR1012 plasmid or the TSA-1-expressing constructs VR1012 TSA1.7 and VR1012 TSA2.1. (A) Immune SCs were obtained 2 weeks after the second immunization and stimulated in vitro with peptide TSA-$1_{515-522}$ (1 μM). After 6 days, recall CTL activity of undepleted (Undepl.) responder cultures was assessed in a 5-h ^{51}Cr release assay against RMA-S ($H\text{-}2^b$) and P815 ($H\text{-}2^d$) target cells sensitized with TSA-$1_{515-522}$ peptide (0.05 μM) at the indicated E/T ratios. RMA-S cells pulsed with $OVA_{257-264}$ peptide (0.05 μM) were used as negative-control target cells. CTL activity of effector cells depleted (Depl.) of $CD4^+$ or $CD8^+$ T cells was measured at a 50:1 E/T ratio against TSA-$1_{515-522}$-sensitized (0.05 μM) target cells. (B) Immune SCs from DNA-vaccinated or *T. cruzi*-infected mice were obtained 7 and 6 months after the second immunization or parasite challenge, respectively, and processed as described for panel A.

This link between parasite load and severity of disease is further supported by the critical role that $CD8^+$ T cells play in parasite control and survival after infection. $CD8^+$ T cells constitute the major component in inflammatory foci of *T. cruzi*-infected tissues (22, 40, 47, 51), and in their absence (52, 54, 55), infected mice have increased mortality rates and tissue parasite loads with a decreased or absent inflammatory response. The recent demonstration of $CD8^+$ CTL in *T. cruzi*-infected mice and humans with a specificity for defined trypomastigote and amastigote surface molecules (32, 61, 62) and of the immunoprotective phenotype that these cells express (61) prompted us to initiate the development of immunization strategies to further characterize the vaccine potential of parasite components known to be targets of protective anti-*T. cruzi* immune responses.

DNA-based immunization has been shown in animal models to easily, safely, and effectively elicit and modulate the spectrum of immune responses necessary for the prevention of

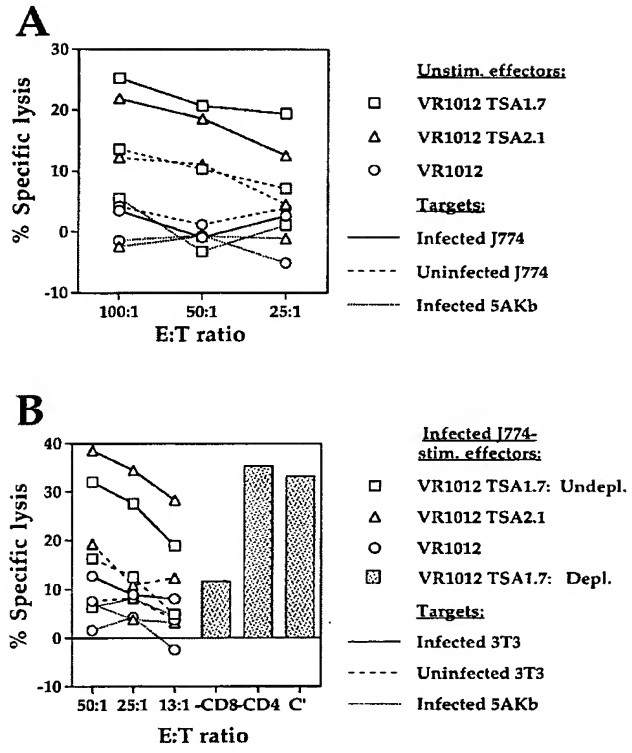

FIG. 4. Induction of parasite-specific, MHC class I-restricted, $CD8^+$-T-cell-dependent CTL response in TSA-1 DNA-immunized BALB/c mice. Two weeks after the second 100-μg intramuscular dose of either VR1012 TSA1.7, VR1012 TSA2.1, or unmodified VR1012 plasmid, immune SCs from BALB/c ($H\text{-}2^d$) mice were prepared. (A) Unstimulated SCs were tested for CTL recognition of *T. cruzi*-infected or uninfected J774 macrophages ($H\text{-}2^d$) and *T. cruzi*-infected 5A.Kb.α3 fibroblasts ($H\text{-}2^k$; $H\text{-}2K^b$) in a 5-h ^{51}Cr release assay at the indicated E/T ratios. (B) Following a 6-day stimulation period with irradiated *T. cruzi*-infected J774 macrophages, effector cells were assayed at the indicated E/T ratios for CTL activity on ^{51}Cr-labeled *T. cruzi*-infected or uninfected 3T3 fibroblasts ($H\text{-}2^d$) and *T. cruzi*-infected 5A.Kb.α3 cells. Effector cells depleted (Depl.) of $CD4^+$ or $CD8^+$ T cells were tested for CTL activity at a 50:1 E/T ratio against *T. cruzi*-infected 3T3 cells. Levels of infection in stimulator cells and target cells ranged from 65 to 75%.

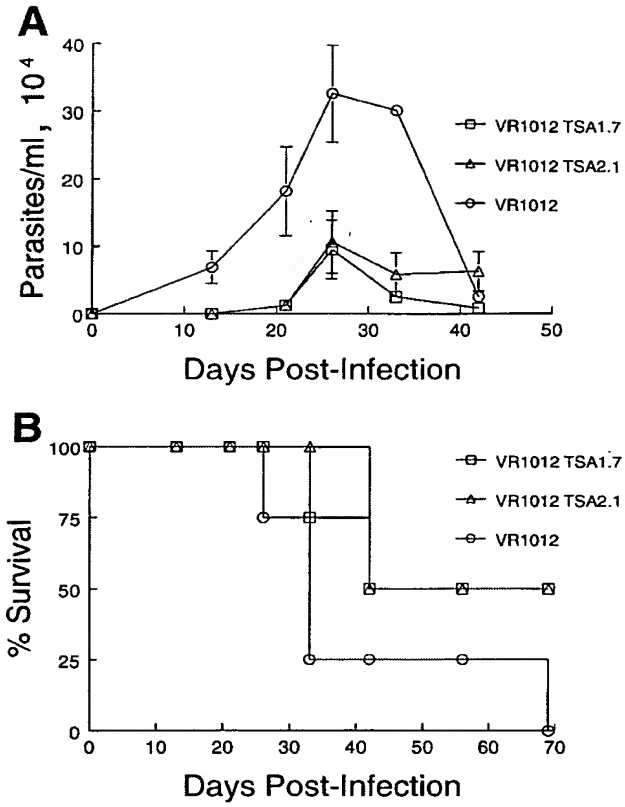

FIG. 5. Parasitemia and protection from *T. cruzi*-induced lethality in TSA-1 plasmid DNA-vaccinated B6 mice. Mice were injected intramuscularly with 100 μg of VR1012 TSA1.7, VR1012 TSA2.1, or control VR1012 plasmid at 0 and 4 weeks, followed 2 weeks later by intraperitoneal infection with 10^5 *T. cruzi* (Brazil strain) BFT. (A) Blood parasite levels in individual mice were monitored by using hemacytometer counts in 10 μl of tail vein blood diluted in an ammonium chloride lysing solution. Values represent mean ± standard errors of the means for surviving mice. (B) Mortality of vaccinated B6 mice infected with *T. cruzi*.

infectious diseases (17, 34, 50, 59, 69, 70) and for the treatment of neoplastic (10, 24, 49), allergic (23, 39), and autoimmune (60) disorders. Thus, we chose this vaccination method to induce *T. cruzi*-specific antibody and class I-restricted $CD8^+$ CTL responses in two inbred mouse strains and to assess its protective efficacy against parasite challenge. Our recent demonstration of TSA-1 as a target of protective CTL (61) made this parasite molecule a prime model antigen to evaluate this immunization method, inasmuch as (i) the N-proximal portion of TSA-1 had already been shown to induce antibody responses which correlate with survival after lethal *T. cruzi* infection (66) and (ii) TSA-1 is a member of the large 85-kDa family of trypomastigote surface proteins which are recognized by human sera and rodent-derived protective antibodies (2, 37).

Plasmid DNA vaccines VR1012 TSA1.7 and VR1012 TSA2.1 were constructed to drive the expression of products TSA-1_{78-652} and TSA-1_{78-790}, which are truncated at the N terminus by 77 residues and at the C terminus by 183 and 45 amino acids, respectively. The main reasons for such a design were twofold: first, because removal of the N-terminal endoplasmic reticulum translocation signal sequence would ensure the cytoplasmic retention of de novo-synthesized TSA-1 protein, its subsequent cytosolic degradation, and an efficient priming of CTL responses; second, because conventional TSA-1 protein-based immunization of BALB/c mice has shown that the C-proximal portion encompassing residues 618 to 835 contains epitopes which interfere with the generation of antibodies to the protective determinants within residues 78 to 619 of the N-proximal portion (66).

Both VR1012 TSA-1 constructs directed the in vitro expression of cytoplasmically retained products with immunoreactivity to sera from *T. cruzi*-infected mice, and in BALB/c mice, both TSA-1-encoding vectors, with and without the repeat sequence, elicited parasite-specific antibody responses. Such responses were detected after the priming dose, and a modest boosting was achieved after the second dose with the VR1012 TSA2.1 vector. By contrast, in B6 mice, parasite-specific antibodies were detected only after the second dose of the VR1012 TSA2.1 vector alone. Similar strain-dependent variability in the induction of antibody responses following plasmid DNA immunization has been reported for the *Plasmodium yoelii*

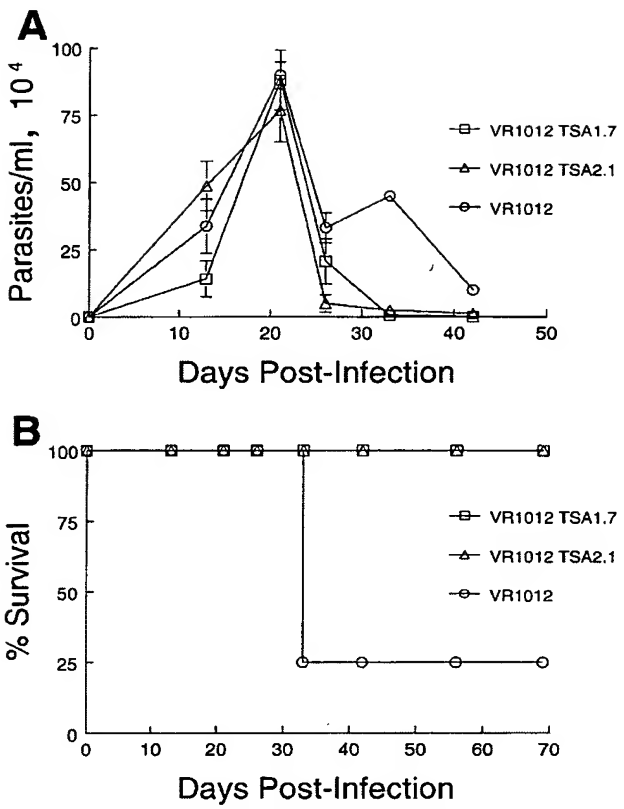

FIG. 6. Parasitemia and protection from *T. cruzi*-induced lethality in TSA-1 plasmid DNA-vaccinated BALB/c mice. Mice were intramuscularly primed and boosted 4 weeks later with 100 μg of VR1012 TSA1.7, VR1012 TSA2.1, or control VR1012 plasmid. After 2 weeks, immunized animals were infected by intraperitoneal injection with 10^3 *T. cruzi* (Brazil strain) BFT. (A) Blood parasite levels were monitored as described for Fig. 5A. (B) Mortality of vaccinated BALB/c mice infected with *T. cruzi*.

TABLE 1. Protection against lethal *T. cruzi* challenge conferred by DNA vaccination[a]

Plasmid DNA	B6 mice		BALB/c mice	
	No. of survivors/ no. challenged	% Survival	No. of survivors/ no. challenged	% Survival
VR1012	0/3		0/3	
	0/4		1/4	
	1/4		0/4	
Total	1/11	9	1/11	9
VR1012 TSA1.7	3/3		3/3	
	2/4		4/4	
	3/4		3/4	
Total	8/11	73	10/11	91
VR1012 TSA2.1	2/3		2/3	
	2/4		4/4	
	2/4		ND[b]	
Total	6/11	55	6/7	86

[a] Mice were primed intramuscularly with 100 μg of VR1012 TSA1.7, VR1012 TSA2.1, or control VR1012 plasmid and boosted 4 weeks later with a similar dose of the respective construct. After 2 weeks, immunized animals were infected by intraperitoneal injection with 10^3 *T. cruzi* (Brazil strain) BFT. Percent survival was assessed at day 100 postinfection.

[b] ND, not determined.

circumsporozoite and hepatocyte-erythrocyte proteins (13). This discrepancy, however, might be a reflection of the well-established genetic control of immune responses to *T. cruzi* (58, 67). We are currently seeking to improve the antibody responses to levels comparable to or higher than those generated by *T. cruzi* infection through the immunization with TSA-1-encoding DNA vaccines that codeliver cytokine genes that had been reported to enhance both humoral and cellular immune responses (9, 16, 24, 27, 68).

The fact that immunization with TSA-1-expressing plasmid DNA vaccines efficiently elicited MHC class I-restricted CTL responses in B6 ($H\text{-}2^b$) and BALB/c ($H\text{-}2^d$) mice is notable, inasmuch as prior to these studies, *T. cruzi*-specific $CD8^+$ CTL had been primed only by parasite infection (32, 36, 61) and TSA-1 had been identified only as a CTL target molecule of B6 mice (61). The demonstration with B6 mice that TSA-1 DNA vaccination and *T. cruzi* infection were able to prime $CD8^+$ CTL populations with specificity for the same protective $H\text{-}2K^b$-restricted TSA-$1_{515-522}$ epitope indicated that similar immunogenic peptides are generated when a cell is transiently transfected in vivo or when it is expressed by an infected cell. In agreement with other studies where DNA immunization has been found to elicit long-lasting CTL responses (6, 57), TSA-$1_{515-522}$-specific CTL were still detected 7 months after administration of the last dose of the TSA-1-encoding DNA. The longevity of the response may be explained by the persistence of the plasmid vaccine in vivo (64) or by recent reports which indicate that CTL memory does not require antigen persistence or CD4 T-cell help (1, 11). Regardless of the mechanisms involved, the ability of genetic immunization to maintain a long-lasting response to protective *T. cruzi* CTL epitopes may have significant potential for the development of DNA vaccines capable of preventing or treating an established *T. cruzi* infection.

While the presence of class I-restricted CTL responsive to *T. cruzi*-infected cells has been demonstrated in BALB/c mice (36), their target antigens have not been identified. Hence, in the absence of known TSA-1-derived $H\text{-}2^d$-restricted CTL peptide epitopes, two alternative strategies were used to determine that TSA-1-expressing DNA vaccines had successfully primed parasite antigen-specific CTL responses. In the first strategy, where the CTL assay was performed on immune SCs without in vitro stimulation, significant genetically restricted CTL reactivity against *T. cruzi*-infected target cells was detected. These results suggest the priming of a substantial number of TSA-1-specific CTL precursors of which a large population remain in a state of activation that allows for their direct detection 2 weeks after the last dose of the DNA vaccine. Similar findings on the detection of CTL activity with unstimulated SCs from mice immunized with DNA vaccines have been reported for the Vif and Nef proteins of human immunodeficiency virus type 1 (27) and for the simian virus 40 T antigen (49). In the second strategy, the stimulating and targeting activities of *T. cruzi*-infected cells were used to confirm the specificity and MHC class I-restricted lytic activity displayed by in vitro-expanded CTL precursors. These findings and the fact that the lytic activity was $CD8^+$ T cell dependent indicate that the observed response was T cell and not NK cell mediated and attest to the value of this method of immunization for priming potent MHC class I-restricted CTL responses in vivo.

Perhaps the most significant finding of these studies was that vaccination with TSA-1-expressing plasmid DNA afforded B6

and BALB/c mice significant levels of protection against lethal *T. cruzi* challenge infection. Overall survival rates of B6 mice vaccinated with VR1012 TSA1.7 or VR1012 TSA2.1 were 73 and 55%, respectively. The same constructs furnished BALB/c mice with nearly complete protection, as 91 and 86% of vaccinated animals, respectively, survived *T. cruzi* infection. These results are in sharp contrast to the 9% survival observed for animals immunized with the unmodified VR1012 plasmid for both strains of mice. It should be noted, though, that immunization with the TSA-1-encoding vectors did not prevent recipient mice from getting infected, and DNA-vaccinated mice from both strains developed parasitemias, albeit at different levels. In B6 mice, the number of circulating parasites in test animals was lower than that observed for recipients of the control DNA vaccine, whereas in BALB/c mice, parasitemias were frequently similar in both groups of animals.

The results presented here lay the foundation for DNA immunization as a strategy for the design of anti-*T. cruzi* vaccines. Using TSA-1 as the model antigen, we demonstrated that this type of antigen delivery was efficient in the induction of parasite-specific antibody and CTL responses as well as in providing significant protection in two inbred strains of mice against *T. cruzi*-induced lethality. Work is now in progress to determine whether the simultaneous delivery of plasmids encoding additional parasite antigens (33, 44) and immunomodulatory cytokines (9, 16, 24, 27, 68) can improve protection and induce efficacious immune responses in genetically diverse strains of mice. Such information may provide strong support for the development of DNA-based vaccines that not only might protect humans at risk of infection with *T. cruzi* but also may alleviate or prevent the pathogenic responses characteristic of chronic Chagas' disease by reducing or perhaps eliminating tissue parasites from infected patients.

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